Hematological Changes in Rats Injected with Acetoacetate (35949)

IDREES BHAI, N. NATH, AND M. C. NATH
University Department of Biochemistry, Nagpur, India

The involvement of ascorbic acid and members of B vitamins, such as riboflavin, pyridoxine, folic acid, and vitamin B_{12} in heme biosynthesis are well known (1–4). Lockhead and Goldberg (5) demonstrated the role of ascorbic acid and glutathione in hemoglobin biosynthesis.

Prolonged administration of ketone bodies such as acetoacetate and β-hydroxybutyrate has earlier been reported from this laboratory to cause a multiple deficiency of B vitamins and ascorbic acid (6–11) as well as depletion in glutathione content of blood and liver in animals (12). Preliminary observations indicated that these animals became anemic following the acetoacetate injection for a long period. In view of these findings, hematological changes in acetoacetate injected rats were studied in considerable detail to learn the severity and type of resulting anemia.

Materials and Methods. Forty eight 4-week-old male albino rats (100–125 g of body wt) reared on normal laboratory stock diet were distributed equally into two groups. Sodium salt of acetoacetate (20 mg/100 g of body wt) was injected daily in experimental rats by the intraperitoneal route. Control rats which were pair fed, were similarly injected with sodium lactate (20 mg/100 g of body wt). The injections were continued for 90 days. Sodium acetoacetate was prepared by the method described by Tidwell and Nagler (13).

Experimental Procedure. a. Blood picture. Hemoglobin and packed cell volume were determined every fortnight by standard techniques (14). Tail blood was used for this purpose. Peripheral blood smears were made for differential count and were stained by the usual standard procedures. Bone marrow smears were made after sternal puncture. Films were fixed with methanol and stained for differential count by the methods described by Wintrobe (14) and for iron by the method of Britton (15).

b. Serum iron and unsaturated iron binding capacity. Serum iron levels and serum unsaturated iron binding capacity (UIBC) were determined by the methods of Williams and Canard (16).

c. Glycine-2-^{14}C incorporation into heme and globin. Hemoglobin biosynthesis was studied by the method described by Schapira *et al.* (17). For this purpose three rats from each group were injected with glycine-2-^{14}C (10 μCi/100 g) 2 hr before sacrifice. Rats were killed by decapitation, blood was collected in centrifuge tubes, and plasma was separated by centrifugation at 2000 rpm. Cells were washed thrice with sodium chloride (0.9%) and then hemolyzed with 2 vol of water and brought to pH 9 with 2 *N* ammonia. Heme was separated from globin by adding 10 vol acetone containing 1% HCl, and filtered. The resulting hemin was precipitated by adding acetone; and then acetone was evaporated at 50°. The precipitate was dissolved in pyridine, filtered into copper planchets, and dried under infrared lamp. The resultant heme pyridine complex was used for radioactive measurement in a Nuclear Chicago gas flow counter after correcting for uniform thickness.

Results. Preliminary work showed that injection of sodium lactate did not cause any significant change in blood picture of rats compared to normal control injected with 0.9% saline. Neither saline nor lactate injected rats developed anemia.

Table I represents the blood picture of the experimental rats following the continuous injections of acetoacetate for a period of 90 days. The fall in the hemoglobin concentration was from 14.5 to 10/100 ml and reduction in number of erythrocytes was from 6.0 to 5.00 million/mm^3. There was a 10% reduc-

TABLE I. Blood Picture in Acetoacetate Injected Rats.[a]

Group	Hemoglobin (gm/100 ml)	RBC (million/ mm³)	PCV (%)	MCV ($\mu\mu^3$)	MCH ($\mu\mu$g)	MCHC (%)
Control (na lactate) 20 mg/100 g	14.5 ±1.5	6.0 ±0.5	45.0 ±3.0	75.0 ±5.0	24.1 ±2.0	32.2 ±2.4
Experimental 20 mg/100 g (na acetoacetate)	10.0 ±1.0	5.0 ±0.4	35.0 ±2.0	70.0 ±5.0	20.0 ±1.5	28.5 ±1.3

[a] PCV = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

tion in cell volume. The values for blood constants such as packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in the acetoacetate injected rats are suggestive of microcytic hypochromic type of anemia. The bone marrow of these rats was markedly cellular and normoblastic in appearance. There was a complete absence of stainable iron (sideroblast).

There was a marked reduction (43%) in serum iron level with significant increase in serum total iron binding capacity in experimental rats (Table II). The results of serum iron, together with study of peripheral blood smear and bone marrow, indicate iron deficiency anemia in experimental rats.

The glycine-2-^{14}C incorporation studies indicated marked decrease in heme synthesis. The specific activity of heme, isolated from the blood of acetoacetate injected rats, was markedly less compared to controls, whereas specific activity of globin remained unchanged in these rats (Table III).

Discussion. It has been postulated that the transfer of plasma iron, present as protein complex, to tissue ferritin requires reduction of the ferric form into the ferrous state (18). The incorporation has been found to be stimulated by ATP and ascorbic acid (19). It has been shown that this catalytic reaction is not only carried out by ascorbic acid alone but also by other reducing agents such as GSH and cysteine (18). It was further suggested that these reducing compounds help in the maintenance of iron in the ferrous form and thus increase the utilization of iron for heme biosynthesis by enhancing enzymic

TABLE II. Serum Iron and Serum Iron Binding Capacity in Acetoacetate Injected Rats.[a]

Group	Serum iron (μg/100 ml)	UIBC (μg/100 ml)	TIBC (μg/100 ml)
Control (10)[b] (Na lactate) 20 mg/100 g	200 ± 17[c]	170 ± 12	370 ± 20
Experimental (12)[b] (Na acetoacetate) 20 mg/100 g	115 ± 18	385 ± 30	500 ± 40

[a] UIBC = unsaturated iron binding capacity; TIBC = total iron binding capacity.
[b] Number of animals used.
[c] Standard error of means.

TABLE III. *In Vivo* Incorporation of Glycine-2-^{14}C into Heme and Globin.

Group	Heme (cpm/μmole)	Globin (cpm/μmole)	Heme/globin
Control (Na lactate) 20 mg/100 g	30.4 ± 1.1[a]	15.2 ± 2.00	2.00
Experimental (Na acetoacetate) 20 mg/100 g	12.1 ± 0.9	17.1 ± 1.60	0.70

[a] Indicates standard error of mean from 3 experiments.

reaction as well as by nonenzymatic chelation. Kometani (20) has earlier demonstrated low serum and tissue iron content in ascorbic acid deficiency. Also it is well known that ascorbic acid is involved in several phases of iron transport, namely, in the absorption of iron in the ferrous state, in facilitating the incorporation of iron into heme in the ferrous state, in the combination of iron with apoferritin to form ferritin and in releasing iron from ferritin (21). Depletion of ascorbic acid and GSH levels in acetoacetate-administered animals was earlier shown by workers from this laboratory (22, 12). Therefore, it seems probable that deficiency of ascorbic acid and glutathione in these experimental rats has resulted in decreased utilization and absorption of iron.

Although the deficiency of any of the B vitamins has been reported to result in anemic condition in experimental animals, pyridoxine deficiency is always associated with decreased hemoglobin synthesis (23). The first precursor of heme, σ-aminolevulinic acid, is derived from the condensation of glycine and succinyl CoA in presence of an enzyme σ-Ala synthetase, pyridoxal phosphate being the cofactor. Nath and Shastri (8) have reported the deficiency of pyridoxine in acetoacetate injected rats. Hence in the deficiency of pyridoxine, condensation of glycine and succinyl CoA cannot result; and hence subsequent products in the pathway of porphyrin synthesis cannot be formed and iron will consequently be unutilized for erythropoiesis. It is obvious from these results that anemia in acetoacetate injected rats has resulted from malabsorption and decreased utilization of iron for hematopoiesis.

Summary. Hypochromic microcytic anemia is produced in rats when injected with acetoacetate (20 mg/100 g) for 90 days. There was a moderate fall in erythrocyte counts, packed cell volume, and hemoglobin levels following the injections of acetoacetate. The anemia induced was most probably due to the deficiency of iron, as well as its malutilization for hemoglobin synthesis. The decrease in hemoglobin synthesis was attributed to multiple deficiency of B vitamins, specially pyridoxine, whereas defect in iron transport and utilization could be due to deficiency of ascorbic acid and glutathione.

The authors gratefully acknowledge the financial assistance from the Council of Scientific and Industrial Research, India.

1. Goldberg, A., Proc. Int. Congr. Soc. Haematol., 7th, Rome **2**, 301 (1958).
2. Penar, O. I., Akad. Med. Nauk USSR **199**, 21 (1959).
3. Mulgaonkar, A. J., and Srinivasan, A., Ann. Biochem. Exp. Med. **19**, 229 (1959).
4. Schulman, M. P., and Richart, C. A., J. Biol. Chem. **181**, 226 (1957).
5. Lockhead, A. C., and Goldberg, A., Biochem. J. **78**, 146 (1961).
6. Nath, M. C., and Chakarbarti, C. H., Ind. J. Med. Res. **41**, 257 (1953).
7. Nath, M. C., and Chakarbarti, C. H., Proc. Soc. Exp. Biol. Med. **82**, 5 (1953).
8. Nath, M. C., and Shastri, N. V., Brit. J. Nutr. **23**, 705 (1969).
9. Nath, N., and Nath, M. C., Proc. Soc. Exp. Biol. Med. **124**, 210 (1967).
10. Sivakumar, B., and Nath, M. C., Curr. Sci. **38**, 110 (1969).
11. Nath, M. C., and Brahmachari, H. D., Nature (London) **157**, 336 (1946).
12. Illing, E. K. B., Gray, C. H., and Lawrence, R. D., Biochem. J. **48**, 637 (1951).
13. Tidwell, H. C., and Nagler, M. E., J. Biol. Chem. **201**, 727 (1953).
14. Wintrobe, M. M., "Clinical Hematology," 6th ed., p. 29. Lea and Febiger, Philadelphia (1968).
15. Britton, C. G. C., *in* "Disorders of Blood," p. 734. Churchill, London (1949).
16. Williams, C. H., and Canard, M. E., J. Lab. Clin. Med. **67**, 171 (1966).
17. Schapira, G., Dryfus, J. C., and Kruh, A., J. Biol. Chem. **82**, 290 (1961).
18. Lockhead, A. C., and Goldberg, A., Lancet **2**, 2711 (1959).
19. Goldberg, A., Brit. J. Haematol. **5**, 150 (1950).
20. Kometani, W., Osaka Daigaku Igaku Zasshi **11**, 1855 (1959).
21. Bothwell, T. H., Jacobes, P., and Charlton, R. W., Brit. J. Haematol. **10**, 50 (1964).
22. Nath, M. C., and Chakarbarti, C. H., Proc. Soc. Exp. Biol. Med. **86**, 851 (1954).
23. Richert, D. A., Pixley, B. Q., and Schulman, M. P., J. Nutr. **71**, 289 (1960).

Received Dec. 1, 1970. P.S.E.B.M., 1971, Vol. 138.